PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, 15/56, 5/14 C12N 9/24, C12Q 1/68 A01H 5/00, C07K 13/00	A1	(11) International Publication Number: WO 92/20807 (43) International Publication Date: 26 November 1992 (26.11.92)

(21) International Application Number: PCT/US92/04282

(22) International Filing Date: 21 May 1992 (21.05.92)

(30) Priority data:
704,288 22 May 1991 (22.05.91) US

(71) Applicant: THE SALK INSTITUTE FOR BIOLOGICAL STUDIES [US/US]; 10010 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors: ZHU, Qun ; 4158 Decoro Street, Apartment 21, San Diego, CA 92122 (US). LAMB, Christopher, J. ; 6444 Farley Drive, San Diego, CA 92122 (US).

(74) Agent: REITER, Stephen, E.; Pretty, Schroeder, Brueggemann & Clark, 444 South Flower Street, Suite 2000, Los Angeles, CA 90071 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: PLANT DEFENSE GENE(S) AND REGULATORY ELEMENT(S)

(57) Abstract

Novel chitinase gene, and its associated regulatory region, from a monocotyledon plant is described.

Bao, et al.
App. No. 09/923,844

REF
A19

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

PLANT DEFENSE GENE(S) AND REGULATORY ELEMENT(S)

The present invention relates to regulatory elements functional in plants, especially monocotyledons. In addition, the present invention relates to novel plant genes encoding products involved in plant defense.

BACKGROUND OF THE INVENTION

The response of plants to microbial attack involves de novo synthesis of an array of proteins designed to restrict the growth of the pathogen. These proteins include hydroxyproline-rich glycoproteins, proteinase inhibitors, enzymes for the synthesis of phytoalexins, enzymes contributing to the reinforcement of cell walls, and certain hydrolytic enzymes such as chitinase and glucanase.

Plant defenses can also be activated by elicitors derived from microbial cell walls and culture fluids. In dicotyledonous plants, extensive studies have shown that microbial attack or elicitor treatment induces the transcription of a battery of genes encoding proteins involved in these defense responses, as part of a massive switch in the overall pattern of gene expression. The functional properties of the promoters of several of these dicotyledonous defense genes have been characterized. In contrast, relatively little is known about the inducible defenses in monocotyledonous plants, including the major cereal crops. For example, the transcriptional regulation of defense genes from monocotyledonous plants has not been examined.

Chitinase (EC 3.2.1.14) catalyzes the hydrolysis of the ß-1,4 linkages of the N-acetyl-D-glucosamine polymer chitin. Chitin does not occur in higher plants, but is present in the cell walls of many fungi. Chitinase, which exhibits complex developmental and hormonal regulation, has been found in many species of higher plants. In addition,

chitinase activity is markedly increased by wounding, ethylene, or microbial elicitors. Furthermore, chitinase is involved in the hypersensitive resistance response to microbial attack. Purified plant chitinase attacks and partially digests isolated cell walls of potentially pathogenic fungi. It is this latter enzyme activity, rather than chitin-binding lectin activity, that is responsible for the inhibition of fungal growth. Chitinase and ß-glucanase exhibit synergistic antifungal activity in vitro. A number of pathogenesis-related proteins (also referred to as "PR proteins") have been found to be chitinases or glucanases.

Chitinase genes from a number of dicotyledonous plants (including bean, cucumber, potato, and tobacco) have been isolated and characterized.

Plant chitinases can be divided into at least three classes, based on amino acid sequence and cellular localization. Class I chitinases are basic isoforms which are structurally homologous and are primarily localized in the central vacuole. Basic chitinases contain a catalytic domain, and a cysteine-rich domain similar to rubber hevein. The hevein domain is thought to serve as an oligosaccharide-binding site. There is a variable spacer region between the hevein and the catalytic domains.

Class II chitinases are usually found in the extracellular fluid of leaves and in the culture medium of cell suspensions, suggesting that they are localized in the apoplastic compartment, consistent with a major function in defense. This hypothesis is supported by recent observations that some PR proteins are acidic chitinases.

Class III chitinases, such as a recently described cucumber chitinase, show no homology with either Class I or Class II chitinases, but are homologous to a

lysozyme/chitinase from Parthenocissus quinquifolia. Class III chitinases are located in the extracellular compartment.

While chitinases from dicotyledons have been well characterized, and many of the corresponding genes have been isolated, there is little information available on the structure and expression of chitinase genes from monocotyledons.

SUMMARY OF THE INVENTION

In accordance with the present invention, we have isolated and characterized a monocotyledon chitinase gene and its associated regulatory sequences. The regulatory sequences of the invention are highly expressed in certain floral organs, and are highly inducible from a low basal level of expression upon exposure to plant defense elicitors.

The regulatory sequences of the invention are useful, for example, for the controlled expression of a wide variety of gene products, such as reporter constructs, functional proteins (e.g., enzymes), and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 presents a comparison of the amino acid sequences of the invention chitinase (derived from rice) with the amino acid sequences of basic chitinases from dicotyledon plants. The predicted amino acid sequence of RCH10 is shown on the top line, while amino acid sequences of tobacco, potato, and bean basic chitinases are aligned with the RCH10 sequence. Only amino acids differing from the RCH10 sequence are shown. "Dots" indicate gaps in the sequence comparison; while an "*" indicates a stop codon.

Figure 2 presents a comparison of the amino acid sequence of the RCH10 hevein domain with the amino acid sequences of the hevein domains of other proteins, i.e., rubber hevein [amino acid residues 1-43; see Lucas et al., FEBS Lett. 193: 208-210 (1985)], potato WIN1 and WIN2 [amino acid residues 26-68 of each; see Stanford et al., Mol. Gen. Genet. 215: 200-208 (1989)], wheat germ agglutinin isolectin [WGA, amino acid residues 88-127; see Wright et al., Biochemistry 23: 280-287 (1984)], rice RCH10 (amino acid residues 22-92), bean basic chitinase [amino acid residues 1-79; see Broglie et al., Proc. Natl. Acad. Sci. USA 83: 6820-6824 (1986)], tobacco basic chitinase (amino acid residues 1-87); tobacco PR-P and PR-Q proteins (amino acid residues 25-57 of each) [see Payne et al., Proc. Natl. Acad. Sci. USA 87: 98-102 (1990) with respect to each of the tobacco sequences]. Each of the above sequences were aligned to maximize sequence identity; only amino acids which differ from the rubber hevein sequence are set forth in the Figure.

Figure 3 summarizes expression results with RCH10-GUS gene fusions in transgenic tobacco plants. Fig. 3A deals with wound and elicitor induction in leaf tissue; Fig. 3B deals with developmental expression in vegetative organs; and Fig. 3C deals with developmental expression in floral organs.

Figure 4 presents the kinetics of wound and elicitor induction of RCH10-GUS gene fusions in transgenic tobacco leaves. Fig. 4A presents results using a substantially intact promoter (including nucleotides -1512 to +76, with respect to the transcription start site; also presented as nucleotides 374 - 1884 of Sequence ID No. 1, plus nucleotides 1 - 76 of Sequence ID No. 2; referred to as construct BZ4-1); Fig. 4B presents results with a deleted promoter (including only nucleotides -160 to +76, with respect to the transcription start site; also

presented as nucleotides 1724 - 1884 of Sequence ID No. 1, plus nucleotides 1 - 76 of Sequence ID No. 2; referred to as construct BZ10-1). Open circles designate wounded leaves, while closed circles designate wounded leaves which have also been exposed to elicitor.

Figure 5 summarizes results of RCH10-GUS gene fusions containing 5' deletions to nucleotide 1724 (designated as -160 in the Figure, i.e., -160 nucleotide upstream of the translation start site), nucleotide 1810 (designated as -74 in the Figure) and nucleotide 1854 (designated as -30 in the Figure) in transgenic tobacco plants. Panel (A) illustrates wound and elicitor induction of RCH10 promoter deletions in mature leaf tissue. Panel (B) illustrates expression in floral organs. Data are presented as mean GUS activities from replicate determinations with extracts from 3 independent BZ10 (-160) transformants, 14 BZ84 (-74) transformants and 10 BZ10 (-30) transformants.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there is provided a DNA fragment comprising a monocotyledon promoter characterized as being responsive to physical and/or biological stress; wherein said DNA fragment is further characterized by the following relative pattern of expression in mature plants:

a low level of expression in leaves;

a moderate level of expression in plant stems; and

the highest level of expression in the plant roots and in the male and female parts of plant flowers.

In accordance with another embodiment of the present invention, there are provided DNA construct(s)

comprising the above-described monocotyledon promoter, operatively linked to at least one structural or functional gene, e.g., a reporter gene.

In accordance with yet another embodiment of the present invention, there is provided plant material transformed with the above-described DNA construct(s).

In accordance with still another embodiment of the present invention, there is provided a method for inducing the expression of heterologous, functional gene(s) in monocotyledon and dicotyledon plants, said method comprising:

subjecting the above-described plant material to conditions which induce transcription of said DNA construct(s).

In accordance with a further embodiment of the present invention, there are provided substantially pure proteins having in the range of about 300 up to 350 amino acids, characterized by:

a hevein domain having in the range of about 40 up to 80 amino acids, wherein said hevein domain is about 70% homologous with respect to dicotyledonous chitinase hevein domains;

a glycine- and arginine-rich spacer region having in the range of about 6 up to 12 amino acids; and

a catalytic domain having in the range of about 240 up to 280 amino acids, wherein said catalytic domain is about 77% homologous with respect to dicotyledenous chitinase catalytic domains.

Proteins of the present invention can optionally further comprise a signal peptide having in the range of about 16 up to 30 amino acids.

A presently preferred protein of the invention has about 336 amino acids, wherein:

the hevein domain has about 40 amino acids;

the glycine- and arginine-rich spacer region has about 12 amino acids; and

the catalytic domain has about 262 amino acids.

This presently preferred peptide will optionally have a signal peptide of about 21 amino acids.

In accordance with a still further embodiment of the present invention, there are provided DNA sequences encoding the above-described protein, optionally further containing a readily detectable label.

In accordance with yet another embodiment of the present invention, there is provided a method for the identification of novel chitinase genes, said method comprising

probing a nucleic acid library with at least a portion of the above-described labeled DNA under suitable hybridization conditions, and

selecting those clones of said library which hybridize with said probe.

The DNA fragment comprising a monocotyledon promoter contemplated by the present invention is responsive to physical and/or biological stress. As used herein, the term "responsive to physical and/or biological stress" refers to DNA sequences which are responsive to exposure to physical stress, such as, for example, wounding (e.g., tearing, folding, bending, and the like), bruising, and the like; or to biological stress, such as, for example, plant defense elicitors (e.g., the high molecular weight fraction heat-released from the cell walls of the soybean fungal pathogen Phytophthira megasperma f. sp. glycinea, purified glucan elicitors, and the like); and so

forth.

The relative expression pattern of peptides maintained under the expression control of the invention monocotyledon promoter in mature plants is typically as follows:

a low level of expression in leaves;

a moderate level of expression in plant stems; and

the highest level of expression in the plant roots and in the male and female parts of plant flowers.

The monocotyledon promoter of the present invention can be further characterized by reference to the sequences set forth in the Sequence Listing provided herewith, referring specifically to Sequence ID No. 1 (and Sequence ID No. 2). For example, a DNA fragment having substantially the same sequence as nucleotides 1836 to 1884, as set forth in Sequence ID No. 1, is operative to confer responsiveness to physical and/or biological stress on a gene associated therewith. Of course, those of skill in the art recognize that longer fragments from the upstream portion of the invention chitinase gene can also be used, such as, for example, a DNA fragment having substantially the same sequence as nucleotides 1810 to about 1884, as set forth in Sequence ID No. 1; a DNA fragment having substantially the same sequence as nucleotides 1724 to about 1884, as set forth in Sequence ID No. 1; a DNA fragment having substantially the same sequence as nucleotides 1558 to about 1884, as set forth in Sequence ID No. 1; a DNA fragment having substantially the same sequence as nucleotides 372 to about 1884, as set forth in Sequence ID No. 1; a DNA fragment having substantially the same sequence as nucleotides 1 to about 1884, as set forth in Sequence ID No. 1; and the like.

In addition, sequences downstream of the transcription start site can also be included in the regulatory elements employed herein (up to about 100 or more nucleotides derived from downstream of the transcription start site can be employed). Thus, the above-described regulatory elements can be extended to comprise, for example, nucleotides 1 - 76 as set forth in Sequence ID No. 2, thereby forming regulatory constructs such as:

a contiguous sequence of nucleotides comprising nucleotides 1836 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

a contiguous sequence of nucleotides comprising nucleotides 1810 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

a contiguous sequence of nucleotides comprising nucleotides 1724 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

a contiguous sequence of nucleotides comprising nucleotides 1558 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

a contiguous sequence of nucleotides comprising nucleotides 372 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

a contiguous sequence of nucleotides comprising nucleotides 1 to 1884, as set forth in Sequence ID No. 1, plus nucleotides 1 - 76 as set forth in Sequence ID No. 2;

and the like.

The monocotyledon promoter of the present invention can be used for the controlled expression (with

respect to both spatial and temporal expression) of a wide variety of gene products. For example, promoter plus reporter constructs (e.g., wherein said reporter gene is selected from chloramphenicol acetyltransferase, β-glucuronidase, β-lactamase, firefly luciferase, and the like) can be used to monitor when and where expression from the invention promoter is induced in a host plant or plant cell.

Alternatively, constructs comprising the monocotyledon promoter of the present invention, plus structural gene, can be employed for the controlled expression of numerous structural (or functional) genes, such as, for example, the Bacillus thuringensis toxin gene, genes encoding enzymes involved in phytoalexin biosynthesis, proteinase inhibitor genes, lytic enzyme genes, genes encoding inducers of plant disease resistance mechanisms, and the like.

Plants contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. Monocotyledons are presently preferred because the invention monocotyledon promoter is expected to be functional in nearly all monocotyledons, whereas dicotyledon promoters have frequently been non-operative when used in monocotyledon hosts. Conversely, it is expected that the invention monocotyledon promoter(s) will be functional in many dicotyledon hosts.

Exemplary monocotyledons contemplated for use in the practice of the present invention include rice, wheat, maize, sorgham, barley, oat, forage grains, as well as other grains.

Plants or plant cells containing the above constructs (introduced by standard techniques, such as, for example, by transfection) can be used to study patterns of development, for the controlled expression of various plant defense genes, for the expression of selectable marker genes (to screen for mutants or compounds that modulate stress signal transduction pathways), and the like.

In accordance with one embodiment of the present invention, the rice chitinase structural gene has also been isolated and characterized. This gene is characterized as having only coding sequence (i.e., contains no introns), and encodes the above-described polypeptide, plus signal sequence. The rice chitinase structural gene can be further characterized as having substantially the same nucleic acid sequence as nucleotides +55 through +1062, as set forth in Sequence ID No. 2.

The rice chitinase gene of the present invention encodes a novel protein, i.e., rice basic chitinase. The rice basic chitinase of the present invention can be further characterized as having substantially the same amino acid sequence as amino acids 22 - 357, as set forth in Sequence ID Nos. 2 and 3 (for the mature form of rice basic chitinase) or amino acids 1 - 357, as set forth in Sequence ID Nos. 2 and 3 (for the precursor-form of rice basic chitinase).

Optionally, the rice chitinase structural gene, or a fragment of at least 100 contiguous nucleotides thereof, can be labeled (wherein said label is selected from a radiolabeled molecule, a fluorescent molecule, a chemiluminescent molecule, an enzyme, a ligand, a toxin, a selectable marker, etc). The resulting labeled rice chitinase structural gene (or a portion thereof) can be used, for example, as a probe (e.g., as part of a method to identify additional monocotyledon or dicotyledon

chitinase-like genes), and the like.

One of skill in the art can readily determine suitable hybridization conditions for screening libraries in search of additional monocotyledon or dicotyledon chitinase-like genes. For example, one would preferably use stringent hybridization conditions when screening for other monocotyledon chitinase or chitinase-like genes; while one would likely use milder hybridization conditions when screening for dicotyledon chitinase or chitinase-like genes. Stringent hybridization conditions comprise a temperature of about 42°C, a formamide concentration of about 50%, and a moderate to low salt concentration. More mild hybridiation conditions comprise a temperature below 42°C, formamide concentrations somewhat below 50%, and moderate to high salt concentrations. Exemplary mild hybridization conditions comprise a temperature of about 37°C, a formamide concentration of about 20%, and a salt concentration of about 5X standard saline citrate (SSC; 20X SSC contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0). Such conditions will allow the identification of sequences which have a substantial degree of similarity with the probe sequence, without requiring perfect homology for the identification of a stable hybrid. The phrase "substantial similarity" refers to sequences which share at least 50% homology. Preferably, hybridization conditions will be selected which allow the identification of sequences having at least 70% homology with the probe, while discriminating against sequences which have a lower degree of homology with the probe.

In the invention method for inducing gene expression in monocotyledon (and dicotyledon) plants, plant material containing DNA constructs under the expression control of invention monocotyledon regulatory sequences is subjected to conditions which induce transcription of the DNA construct. Such conditions include exposing the plant

or plant material to physical stress (e.g., wounding) and/or biological stress (e.g., infection, elicitor molecules derived from pathogens).

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Nucleotide sequences were determined by the dideoxy chain-termination [Sanger et al., PNAS 74: 5463-5467 (1977)]. Fragments for sequencing were obtained by restriction endonuclease digestion or exonuclease III deletion [Ausubel et al., Current Protocols in Molecular Biology, Wiley, NY (1987)].

EXAMPLE I
Plant Material

Rice (Oryza sativa L. cv. IR36) seeds were sterilized in 70% ethanol for 2 minutes and then in a 2% solution of sodium hypochlorite for 30 minutes. Sterilized seeds were germinated and grown in MS medium (without hormones) in darkness [Murashige and Skoog, Physiol. Plant 15: 473-497 (1962)]. Two weeks after germination, leaves, roots and stems were harvested separately, then immediately frozen in liquid nitrogen and stored at -80°C until required. Rice (cv. CR76) cell suspension cultures were grown in N6 medium [Chu et al., Scientia Sinica 5: 659-668 (1975)] and maintained in darkness. The high molecular weight fraction heat-released from mycelial cell walls of Phytophthora megasperma pv. glycinea (Pmg) was used as elicitor [Sharp et al., J. Biol. Chem. 259: 11321-11326 (1984)]. Elicitation experiments were conducted on 5-day-old cultures, the stage of the cell culture cycle during which maximum responsiveness to elicitor was observed.

EXAMPLE II
DNA and RNA Isolation

Genomic DNA from rice cell suspension cultures was prepared according to the method of Ausubel et al., supra. DNA was isolated from tobacco leaves as described by Schmid et al., Plant Cell 2: 619-631 (1990). Plasmid and phage DNA were isolated by standard methods [Maniatis et al., Molecular Cloning: A laboratory manual, Cold Springs Harbor Laboratories, Cold Spring Harbor, NY (1982)]. RNA from cell suspension cultures and plant tissues was prepared by the guanidinium isothiocyanate method [Chomczynski and Sacchi, Anal. Biochem. 162: 156-159 (1989)].

EXAMPLE III
Isolation and Characterization of Genomic Rice Clones

A lambda-DASH library containing 15-25 kb genomic fragments from a Sau3A partial digest of rice genomic DNA was a gift from N.H. Chua. pCht12.3, a 650 bp bean basic chitinase cDNA fragment cloned in pBluescript, was used as probe [Hedrick et al., Plant Physiol. 86: 182-186 (1988)]. For library screening, filters were pre-hybridized for 2-4 hours at 42°C in 30% formamide, 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 5 x SSC (1 x SSC is 0.15 M NaCl, 15 mM sodium citrate), and 100 μg of sheared salmon sperm DNA per ml. The filters were then hybridized for 24 hours at 42°C in the same buffer with nick-translated probe DNA. Filters were washed in 2 x SSC, 2% SDS at 42°C for 30 minutes and autoradiographed at -80°C. Purified phage clones containing chitinase sequences were analyzed by restriction endonuclease digestion and Southern blot hybridization. Selected restriction fragments were subcloned into pGEM7 or pBluescript vector.

EXAMPLE IV
DNA Blot Hybridization

Rice genomic DNA samples were digested with various restriction enzymes, fractionated by electrophoresis on a 1% agarose gel and blotted onto a nylon membrane (Genescreen plus). Hybridization to genomic DNA was performed for 24 hours at 65°C in 1% SDS, 1M NaCl, 10% dextran sulfate, 100 μg per ml sheared, denatured salmon sperm DNA, and the DNA probe labeled with [^{32}P]. The membrane was washed with constant agitation, twice in 2 x SSC for 5 minutes at room temperature and once in 2 x SSC, 1% SDS for 45 minutes at 65°C.

Genomic Southern blots with tobacco DNA were probed with the HindIII/SacII fragment of pBI101 containing GUS coding sequences using standard procedures.

EXAMPLE V
RNA Blot Hybridization

RNA samples were separated by electrophoresis on a 1% agarose formamide gel in 1 x 3-[N-morpholino]-propanesulfonic acid (MOPS)/EDTA buffer (10 x MOPS/EDTA buffer is 0.5 M MOPS, pH 7.0, 0.01 M EDTA, pH 7.5), and blotted onto a nylon membrane. Before hybridization, the membranes were baked at 80°C for 2 hours. The same hybridization conditions as in Southern blot analysis were used, except that hybridization was at 60° instead of 65°C.

EXAMPLE VI
Fusion Protein Analysis

A 941 bp fragment from the chitinase RCH10 coding region (positions +85 to +1026 relative to the transcription start site; nucleotides 85 - 1026, see Sequence ID No. 2) was inserted into pRX-1, pRX-2, and pRX-

3 expression vectors [Rimm and Pollard, Gene 75: 323-327 (1989)] to generate pBZ7-1, pBZ7-2, and pBZ7-3, respectively. These plasmids were transferred into Escherichia coli strain HB101 by the $CaCl_2$ method [Maniatis et al. supra], and the transformed cells grown to stationary phase at 37°C in LB broth. The cells were then inoculated into 5 ml of M9-CA minimal medium containing 100 μg/ml ampicillin, grown for 3 hours at 37°C, and then induced by addition of indolylacrylic acid to a final concentration of 10 μg/ml. After 5 hours, the cells were harvested and lysed by sonication in 10 mM TRIS-HCl, pH 8.0, 50 mM EDTA, 8% sucrose, 0.5% Triton X-100, and lysozyme (2 mg/ml). Soluble bacterial extracts were analyzed in a 10% SDS-polyacrylamide gel [Maniatis et al. supra]. Immunoblotting was performed as described by Bradley et al., Planta 173: 149-160 (1988). Antiserum to bean chitinase, prepared employing standard techniques, was obtained as a gift from T. Boller.

EXAMPLE VII

Isolation and Nucleotide Sequence of RCH10

A rice genomic library was screened using as a probe the insert of pCht12.3, which contains cDNA sequences of a bean basic chitinase [Hedrick et al., supra]. From 12 plaque-purified clones, 3 positive clones were characterized by restriction mapping and Southern blot hybridization. A 2.5 kb HindIII fragment from one of these clones, designated RCH10, was subcloned. Nucleotide sequencing showed that this fragment contained a 1.0 kb open reading frame (ORF), together with 1.5 kb of upstream sequence. Subcloning of two HincII fragments that overlapped the HindIII fragment gave an additional 372 bp of nucleotide sequence 5' of the HindIII fragment and 125 bp 3' of this fragment. This 3.0 kb sequence contained the complete RCH10 chitinase gene (see Sequence ID No. 1).

A single long ORF with no introns encoded a polypeptide of 336 amino acids (see Figure 1 and Sequence ID No. 2). Figure 1 shows the primary structure of the RCH10 gene product compared with basic chitinases from dicotyledon plants. The RCH10 polypeptide contains a hydrophobic putative signal peptide of 21 amino acids at the N-terminus, as well as hevein and catalytic domains. The hevein domain of RCH10 is about 40 amino acids long and is cysteine-rich. Figure 2 shows a comparison of the hevein domain of RCH10 with the hevein polypeptide and other gene products containing this domain, including WIN1, WIN2, and wheat germ agglutinin isolectin. The hevein domain of RCH10 shares about 70% amino acid sequence identity with these other hevein domains. The hevein domain and catalytic domain of RCH10 are separated by a glycine- and arginine-rich spacer region. The amino acid sequence identity between the RCH10 catalytic domain and the catalytic domains of chitinases from dicotyledons is about 77%.

EXAMPLE VIII
TrpE-RCH10 Fusion Protein

The level of similarity between RCH10 and basic (class I) chitinase genes from dicotyledons strongly suggests that RCH10 encodes a rice chitinase. To confirm the identity of the protein product encoded by the RCH10 gene, a fragment from the coding region (positions +85 to +1026) was inserted into the E. coli expression vectors pRX1, pRX2, and pRX3 to obtain the plasmids pBZ7-1, pBZ7-2, pBZ7-3. pBZ7-1 codes for a fusion polypeptide consisting of 18 amino acids from TrpE, 3 amino acids from the linker sequence, and 314 amino acids from the chitinase gene fused in the same reading frame. pBZ7-2 and pBZ7-3 are respectively 1 and 2 bases out of frame compared to pBZ7-1. These three plasmids were transferred into E. coli strain HB101, and soluble bacterial extracts were separated in a

10% SDS-poly-acrylamide gel and stained with Coomassie blue. The results showed an additional 37.5 kDa polypeptide in the cells transformed with pBZ7-1, whereas no additional polypeptides were detected in cells transformed with pBZ7-2 or pBZ7-3. Western blot analysis showed that the 37.5 kDa species in cells transformed with pBZ7-1 reacted with antiserum to bean chitinase, confirming that the RCH10 gene encodes a rice chitinase.

EXAMPLE IX
Transcription Start Site

The transcription start site was determined by primer-extension analysis using a synthetic 28-mer oligonucleotide identical to the sequence of the antisense DNA strand at residues 132-104 downstream from the translational initiation codon (5'-CCG-AAC-TGG-CTG-CAG-AGG-CAG-TTG-G-3'). Primer extension analysis was performed by the method of Jones et al., Cell 48: 79-89 (1987), using the synthetic oligonucleotide wherein the 5' terminus was labeled with [^{32}P]. No band was found in the reaction with RNA isolated from control cells, whereas two bands were detected in the reaction with RNA isolated from elicitor-treated cells. The major product was 186 nucleotides in length and corresponded to the position of the first 'A' in the sequence CCCTCAATCT, which closely resembles an eukaryotic transcription initiator sequence [Smale and Baltimore, Cell 57: 103-113 (1989)]. This position was designated as +1. An additional product two nucleotides smaller than the major reverse transcript was also detected. The putative translational initiation codon was 55 bp downstream from the major transcription start site.

EXAMPLE X

Flanking Sequences

Putative TATA and CAAT boxes were located 44 and 75 bp respectively upstream from the transcription start site (see Sequence ID No. 1) The DNA sequence between these two boxes was GC-rich (72%). Two inverted putative GC boxes were present at positions -55 to -60 and -66 to -70 [Kadonaga et al., Trends Biochem. Sci. 11: 20-23 (1986)]. A sequence similar to the binding site for an elicitor-inducible factor in a parsley phenylalanine ammonia-lyase promoter occurred in the inverted orientation at positions -108 to -117 [Lois et al., EMBO J. 8: 1641-1648 (1989)]. An imperfectly duplicated TGTCCACGT motif was located at positions -752 to -736. In vivo footprinting studies have demonstrated constitutive binding of a nuclear factor to this motif [Lois et al., supra). Putative cis-acting elements in the 5' flanking region of RCH10 are summarized in Table 1:

Table 1

Repeat sequences and putative cis-elements in the RCH10 promoter

Class	Position*	Sequence
TATA box	1836 - 1843	TATATAA
CAT box	1806 - 1810	CCAAT
GC box-like motif	1815 - 1819	CGCCC(inverted)
	1824 - 1830	CCCGCGG(inverted)
Elicitor-inducible PAL** footprint	1770 - 1778	TGGCAATGC(inverted)
Constitutive PAL footprint	1133 - 1139	TGTCCAA
	1140 - 1146	TGTCCAC
Direct repeat 1	331 - 343	GTATGTAAAAAG
	363 - 374	GTATGTAAAAAG
Direct repeat 2	748 - 759	TGGGAGCAGCGG
	912 - 923	TGGGAGCAGCGG
Direct repeat 3	1459 - 1473	TACTCTGTGTGATGA
	1494 - 1507	TACT-TGTGTGATGA
Inverted repeat 1	541 - 550	AATTTTTTAA
	1229 - 1238	TTAAAAAATT
Inverted repeat 2	1257 - 1266	TCCCCAAGGT
	1650 - 1659	TGGAACCCCT
Triplicated motif	1723 - 1738	ATGCATGCATATGCAT

* Numbers refer to the sequence presented in Sequence ID No. 1

** PAL = phenylalanine ammonia-lyase

A computer-aided search failed to identify significant sequence homology between the rice RCH10 promoter and the promoter of an ethylene-inducible bean chitinase [Broglie et al., Proc. Natl. Acad. Sci. USA 83: 6820-6824 (1989)]. Two putative polyadenylation signals at positions 1054 (AAATAA; see Sequence ID No. 2) and 1093 (AATAAA; see

Sequence ID No. 2) were found in the 3' flanking region. These sequences fit the consensus polyadenylation sequence (A/GAATAA) described in plants [Heidecker and Messing, Annu. Rev. Plant Physiol. 37: 439-466 (1986)].

EXAMPLE XI
Organization of Rice Chitinase Genes

To estimate the number of chitinase genes in the rice genome, Southern blots of genomic DNA from rice were hybridized with the SacII-HindIII fragment of pRCH10 (positions 422 to 1021; see Sequence ID No. 2), which encodes a region conserved among class I and class II chitinases. This probe hybridized to several restriction fragments of rice genomic DNA digested with EcoRI, ClaI, HindIII or PvuII, indicating the presence of a family of chitinase genes in the rice genome.

EXAMPLE XII
Chitinase Gene Expression in Plants and Elicitor-treated Cell Populations

RNA isolated from rice cell suspension cultures treated with the Pmg fungal elicitor were hybridized with the fragment from the conserved region of the RCH10 gene, and also with an RCH10-specific sequence, the SphI-MluI fragment (positions 114 to 259; see Sequence ID No. 2). A low basal level of chitinase transcripts could be detected in cells of suspension cultures when the fragment from the conserved region was used as probe. However, when the RCH10-specific fragment was used as the probe, no basal level of transcripts was detectable. Thus, the basal level of chitinase transcripts in cells in cultured suspension was not due to RCH10, but represented the expression of other members of the gene family. Following treatment with Pmg elicitor, accumulation of chitinase transcripts could be detected within 2 hours, with maximum levels after 6

hours. Hybridization with the RCH10-specific probe showed a similar marked accumulation of the RCH10 transcript over the time course of 2-6 hours. Northern blot analysis of RNA from different organs showed that transcripts of rice chitinase accumulate to high levels in roots, but only to barely detectable levels in stems and leaves.

EXAMPLE XIII
Construction of Gene Fusions

A 2538 bp HindIII fragment from the RCH10 gene was subcloned into pGEM7, and a HindIII/BalI fragment (a contiguous fragment containing nucleotides 372 - 1884 of Sequence ID No. 1, plus nucleotides 1 - 76 of Sequence ID No. 2) was then inserted into the HindIII/SmaI site of the GUS expression vector pBI101.2 [Jefferson et al., EMBO J 6: 3901-3907 (1987)] to give pBZ4. A 1463 bp HincII fragment from RCH10 was cloned into the pGEM7 SmaI site, and a XbaI/BalI fragment (a contiguous fragment containing nucleotides 1558 - 1884 of Sequence ID No. 1, plus nucleotides 1 - 76 of Sequence ID No. 2) was then inserted into the XbaI/SmaI site of pBI101.2 to give pBZ14. A 276 bp SphI fragment from RCH10 was cloned into pSP72, and a HindIII/BalI fragment (a contiguous fragment containing nucleotides 1724 - 1884 of Sequence ID No. 1, plus nucleotides 1 - 76 of Sequence ID No. 2) was then inserted into the HindIII/SmaI site of pBI101.2 to give pBZ10. The RCH10-GUS translational fusions in pBZ4, pBZ14 and pBZ10 were confirmed by direct double-stranded sequencing using a GUS-specific primer.

EXAMPLE XIV
Tobacco Transformation

pBZ4, pBZ14 and pBZ10 were mobilized from Escherichia coli HB101 into Agrobacterium tumefaciens LBA 4404 [Jefferson et al., supra], and transgenic tobacco plants generated by the leaf disc method [Rogers et al., Methods Enzym. 118:627-640 (1986)]. Transformed plants were selected on Murashige and Skoog medium [Murashige and Skoog, supra] containing 200 μg/ml kanamycin and 500 μg/ml carbenicillin or cefatoxim, and grown at 25°C under a 16-hour light (115 mE)/8-hour dark cycle.

pBZ4 contains the 5' flanking sequence of RCH10 from nucleotide 372 and downstream thereof (i.e., non-coding sequence of 1512 nucleotides), the 55 bp leader sequence and the first 22 bp of the RCH10 coding sequence, fused in frame with the GUS coding sequence in the vector pBl101 [Jefferson et al., supra]. This gene fusion was transferred to tobacco by *Agrobacterium tumefaciens*-mediated leaf disc transformation [Rogers et al., supra] and plants regenerated under kanamycin selection. Of 20 kanamycin resistant plants, 14 exhibited GUS activity in extracts of young leaves. Twelve of these GUS-positive plants were confirmed as transformants containing one T-DNA copy by Southern blot hybridization, and four, designated BZ4-1, BZ4-5, BZ4-7 and BZ4-14, were selected for further studies.

EXAMPLE XV
Wound and Elicitor Induction

Discs (about 8 mm in diameter) excised from fully expanded leaves were incubated in 50 mM sodium phosphate buffer (pH 7.0) at 25°C in the dark. Tissue samples were snap frozen in liquid nitrogen and stored at -80°C. Fungal elicitor was the high molecular weight fraction heat-

released from washed mycelial walls of Phytophthora megasperma f.sp. glycinea [Ayers et al., Plant Physiol. 57: 760-765 (1976)], and was applied to wounded tissue in 50 mM sodium phosphate buffer (pH 7.0) at a final concentration of 100 μg glucose equivalents/ml.

Excision wounding of leaf tissue caused a marked increase in GUS activity. In transformants BZ4-1 and BZ4-14, wounding resulted in 10- to 20-fold increases in GUS activity (relative to the low basal levels of 49 and 22 pmole of product/minute/mg protein, respectively, in unwounded tissue; see Figure 3A). In transformants BZ4-5 and BZ4-7, the levels of GUS activity in unwounded leaves were 920 and 570 pmole/minute/mg protein, and wounding caused a 2- to 3-fold increase in these relatively high basal levels.

Addition of fungal elicitor to the leaf tissue immediately after excision caused a further marked stimulation of the expression of the gene fusion, compared with equivalent excision-wounded tissue not treated with elicitor (see Figure 4A). Increased GUS activity was observed 16 hours after elicitor treatment with maximum levels after 48 hours (see Figure 4A), whereas the response to excision wounding in the absence of elicitor was somewhat slower. Overall, elicitor treatment of excised leaf discs caused a 40- to 60-fold increase in GUS activity over low basal levels in BZ4-1 and BZ4-14 plants, compared with a 4- to 6-fold increase in BZ4-5 and BZ4-7 plants, which exhibited higher basal levels of expression (see Figure 3A).

Histochemical analysis of GUS activity in situ showed that wound induction of the gene fusion was restricted to the tissues immediately adjacent to the wound surface, whereas elicitor also induced expression in tissues at a somewhat greater distance from the wound surface.

Ethylene, administered as ethephon, had no effect on the level of GUS activity in intact leaves.

EXAMPLE XVI

Developmental Expression

In addition to elicitor and wound induction in leaf tissue, the RCH10-GUS gene fusion was also expressed during normal development in the absence of an applied stress. Thus, high levels of GUS were observed in roots and moderate levels in stems compared to the relatively weak expression in young leaves (see Figure 3B). Although there was, as expected, some variation among the independent transformants in the absolute levels of expression, the same overall pattern of GUS activity was observed in each case: root > stem > leaf. Histochemical analysis showed strong expression of RCH10-GUS in juvenile tissue of apical root tips. In stems, GUS staining was localized to the epidermis and vascular system. In the latter, staining was not restricted to specific tissue-types, but was observed in a number of locations including the outer phloem, inner phloem and xylem. No GUS staining was observed in pith or cortical tissue.

The RCH10-GUS gene fusion also exhibited a characteristic pattern of expression in floral organs. Thus while only low levels of GUS activity were observed in sepals and petals, comparable to the levels in leaves from the same plants, relatively high levels were found in anther, stigma and ovary extracts (see Figure 3C). This organ-specific pattern of expression was confirmed by histochemical analysis of GUS activity *in situ*. Moreover, the *in situ* analysis showed that within anthers there was strong expression of the gene fusion specifically in pollen, since no staining was observed with ruptured anthers from which the pollen had been expelled, whereas strong staining was readily detectable with intact anthers

containing mature pollen. GUS activity was also directly demonstrated by histochemical staining of isolated pollen.

EXAMPLE XVII
Promoter Deletions

To localize cis-elements that specify the complex developmental regulation and stress induction of the RCH10 promoter, the expression was analyzed for gene fusions with upstream (i.e., 5') portions of the promoter deleted, e.g., deleted to position 1558 (see Sequence ID No. 1; BZ14); deleted to position 1724 (see Sequence ID No. 1; BZ10); deleted to position 1810 (see Sequence ID No. 1; BZ74); and deleted to position 1854 (see Sequence ID No. 1; BZ30). Ten independent BZ14 transformants and 7 BZ10 transformants were examined, and in both cases two representative plants were analyzed in further detail.

Strikingly, the full pattern of expression established for the BZ4 plants containing the promoter, deleted only to nucleotide 372 (see Sequence ID No. 1), was also observed in plants containing the much more extensive deletions, i.e., BZ14 (deleted to position 1558, refer to Sequence ID No. 1) or BZ10 (deleted to position 1724, refer to Sequence ID No. 1) See Figure 3B. Thus, the BZ14 and BZ10 transformants exhibited wounding and elicitor induction of GUS activity from low basal levels in leaf tissue, with similar fold-inductions over basal levels and similar absolute levels of GUS activity in induced tissue as observed in BZ4 plants containing the full promoter (containing nucleotides 372 to 1884 as presented in Sequence ID No. 1). Likewise, the kinetics for wounding and elicitor induction of the constructs containing substantial promoter deletions (i.e., the 1558 - 1884 and 1724 - 1884 constructs) were the same as with the full promoter. The BZ14 and BZ10 plants also showed the same characteristic pattern of expression in floral organs as

observed with the full promoter, with high levels of GUS activity in anthers, stigmas and ovaries compared to relatively weak expression in sepals and petals (see Figure 3C). In vegetative organs of BZ14 and BZ10 transformants, the levels of GUS activity were: root > stem > leaf, as observed with the full promoter, although the expression in roots and stems was markedly reduced compared to BZ4 plants (see Figure 3B).

In contrast, deletion of the 5'-most 1724 nucleotides (i.e., to -160 nucleotides from the translation start site) caused a marked reduction in the levels of GUS activity in vegetative organs, although the relative expression in different organs was the same as observed with the full promoter: root > stem > leaf. Thus, there appears to be an enhancer element located between nucleotide 1558 and 1724 that is important for expression in vegetative development, but is not required for floral expression or stress induction.

To delineate cis-elements in the proximal region of the promoter, floral expression and stress induction of the RCH10-GUS gene fusion were compared in BZ74 and BZ30 transformants. Fourteen BZ74 and 10 BZ30 transformants were examined. BZ74 (i.e., where 5'-noncoding nucleotides from 1810 and upstream thereof are deleted) transformants still exhibited wounding and elicitor induction of GUS activity from low basal levels in leaf tissue, although the absolute induction was not as high as in BZ10 plants (Figure 5A). However, BZ30 (i.e., where 5'-noncoding nucleotides from 1854 and upstream thereof are deleted) transformants showed no increase in GUS activity in response to wounding and elicitor treatment, indicating the presence of a cis-element for stress induction between nucleotide 1810 and 1854 (Figure 5A). In contrast, deletion of the first 1810 upstream nucleotides abolished expression in floral organs (Figure 5B), indicating the

presence of a distinct cis-element necessary for floral expression but not stress induction located between nucleotide 1724 and 1810.

EXAMPLE XVIII
GUS Assays

GUS activity was assayed in tissue extracts by fluorimetric determination of the production of 4-methylumbelliferone from the corresponding ß-glucuronide [Jefferson et al. supra; Jefferson, Plant Mol. Biol. Rep. 5: 387-405 (1987)]. Root, stem and leaf tissues were collected from 10 cm-tall plantlets and floral organs were collected from mature fully open flowers. Protein was determined by the method of Bradford [Anal. Biochem. 72: 248-254 (1976) and GUS activity was expressed as pmole of product/minute/mg of protein. Histochemical localization of GUS activity in situ was performed with the chromogenic substrate 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc). Stem sections were cut by hand, vacuum-infiltrated with 50 mM sodium phosphate buffer (pH 7.0) containing X-gluc and incubated at 37°C. Flowers and roots were directly incubated in X-gluc solution. After overnight incubation, chlorophyll was removed by immersion of the tissue samples in 70% ethanol prior to examination using a Nikon Diaphot TMD microscope.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SUMMARY OF SEQUENCES

Sequence ID No. 1 is the nucleic acid sequence for a regulatory region (i.e., the upstream or 5'region) of a rice chitinase gene of the invention.

Sequence ID No. 2 is the nucleic acid sequence and deduced amino acid sequence for a rice chitinase gene according to the present invention.

Sequence ID No. 3 is the deduced amino acid sequence for the rice chitinase gene presented in Sequence ID No. 2.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: LAMB, Ph.D., CHRISTOPHER J.
ZHU, Ph.D., QUN

(ii) TITLE OF INVENTION: PLANT DEFENSE GENES AND PLANT DEFENSE REGULATORY ELEMENTS

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: PRETTY, SCHROEDER, BRUEGGEMANN & CLARK
(B) STREET: 444 South Flower Street, Suite 2000
(C) CITY: Los Angeles
(D) STATE: California
(E) COUNTRY: United States
(F) ZIP: 90071-2921

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) PRIORITY APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/704,288
(B) FILING DATE: 22-MAY-1991
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Reiter, Mr., Stephen E.
(B) REGISTRATION NUMBER: 31192
(C) REFERENCE/DOCKET NUMBER: FP41 9322

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (619) 546-7437
(B) TELEFAX: (619) 546-9392
(C) TELEX: 9103330318

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1884 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTTAACTGCC AGCTTCAAAT TATTTATAGA TAATTTAATA GCCAATTCAT CTAATAGTTA 60

TTTATTATAC TATTAATATC TGATCTCACC TGAGTCATAC TACAGCTGGC TACAAATGTG 120

TAGTGTACTA CTCTTTCTCT CTTCTTTTAT CTCTTTAAAA TATGTTATAG CGGCTTATAA 180

CTGTTATTGT ACCTGCTCTA AGTCGATCGT GATGATCGAT CATTCGTCAA ATGTTACCAC 240

GTCCAGTGAC TTATCCATGG TTCACCTTAC TATAAAAAAT GATTTTTATG GACAACTCCT 300

TTAATTTTGT TCAAACGGAC CAAAGAAACC CGTATGTAAA AAGGTTGGGA ATATCTGATC 360

CTGTATGTAA AAAGCTTGGA ATATCTGATA GAGGGCAAAC TTGTGAAAAT TGTTTTTTTA 420

AGATGGACCT CTTAACAAGC CTACTTGCAA AAAATCGACC TATTTACATA GACGGACTTG 480

TTAAGAGACT TGTCTATGAA AATCGGTGGA TAGCATGACC GGTCACAATA CTTCCCCTAT 540

AATTTTTTAA TCCTCCTAGA TAAACCCTAT CTCTCTCTTC ATGTTCTTTG CTTTCCATCT 600

ATAGTCTCGC ATCCCTCATC ACCTCCCATT CCTCTCTCTC TCACCCCCTG CTCAGTGGGA 660

GCGCAGCTGG CGATGGCACC ACCGGCGACA AGAGGGGCCA GAGGCTAGCA TGTGCACGGA 720

AGTGACAATG GCGCCACATG ATTAGCATGG GAGCAGCGGC GCGTTTCATC AGGACACGCT 780

GCAATTGGCT CTAGTGACGG CACCCTTGAG AGGACATGGT AGCGGTGGCG CCTCAGGAGT 840

GGTGGGGCAC GGTGGCAGAA CTCCGGCGGT GGCAAGCCAC CACACAGCGA CAGATCCACC 900

ACCACCGACC TTGGGAGCAG CGGGGCCTCA GCGGTGATGA CGATGGTAGA TCGAAGCTAG 960

GGTTTCTATT TTTTTTTGCT GCAAAAATCA CTTTTTACAC ATGGGTACAT GCATGTTTTT 1020

TACATACACC TAGTATTAGG TGGGCCGTCC ACCCGTTCGC AAAGATCATT TATGCAGTCA 1080

TCATGATCGG AGATGGAACT ATGGAGACAT ATATGCAAGT ATTTGGCCAA CATGTCCAAT 1140

GTCCACCAGA TTGGGAGCTC AATCCTACCC CGTGGTATGG GTATGTTACT GTGCGCCTAA 1200

TATTTACGTA CGCTGGTTTA ATCTATTTTT AAAAAATTTG CTACATACTC CCTCCGTCCC 1260

CAAGGTTGGC TTTTTTTTTT TGGAGGGAGA GAGTAATATT TAGAGTTTGT GGTTTTTGTT 1320

ATTGAACACC TTAAAAGGCA TGAAACGACT TGTCGGAGAA CGAATCTCCT CTAGCAGGGA 1380

AGCAACGAAC CTCCCAAAAA AAACAAAAAA AAACTCCTCC TTTCATGATT CAACCAAAGG 1440

GCAATTTGAG ATCGAGCCTA CTCTGTGTGA TGAACTCAAA ACACAATCAA GTATACTTGT 1500

GTGATGAGCG GTGAGCCAGA TATGTTCCTG CTCTGTCCGT GCTCGACTCA ATTCATTGTC 1560

AACCCTAGCG ATTTCCATTA ATGCAATGAC TATATGAAAT GCAAAGATGT ACTATATGAC 1620

TACTAGTTGG ATGCACAATA GTGCTACTAT GGAACCCCTT TTGCCCCTCT AATAGTAGGA 1680

TCTAGGCTAA ATGACGTTTC AATAAATCAC AGTTAGTAAG GGATGCATGC ATATGCATGA 1740

TATGTGAGTG TCTGTTAATC GTGGCAAATT GGCAATGCAA TTTGTTGTTG AAAAATACCA 1800

AGATGCCAAT ACTACGCCCA CTTCCCGCGG CGCTCTATAT AAAGCCATGC GCTCCCATCG 1860

CTTCTTCCTC ACAAACTTTC CCTC 1884

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1151 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 55..1062

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AATCAGTCAA TCTGTATACA GCAACTCAGC GATCTTATAT TTACCCAACA CACC ATG 57
Met
1

AGA GCG CTC GCT GTG GTG GCC ATG GTG GCC AGG CCC TTC CTC GCG GCG 105
Arg Ala Leu Ala Val Val Ala Met Val Ala Arg Pro Phe Leu Ala Ala
5 10 15

GCC GTG CAT GCC GAG CAG TGC GGC AGC CAG GCC GGC GGC GCG GTG TGC 153
Ala Val His Ala Glu Gln Cys Gly Ser Gln Ala Gly Gly Ala Val Cys
20 25 30

CCC AAC TGC CTC TGC TGC AGC CAG TTC GGC TGG TGC GGC TCC ACC TCC 201
Pro Asn Cys Leu Cys Cys Ser Gln Phe Gly Trp Cys Gly Ser Thr Ser
35 40 45

GAC TAC TGC GGC GCC GGA TGC CAG AGC CAG TGC TCG CGG CTG CGG CGG 249
Asp Tyr Cys Gly Ala Gly Cys Gln Ser Gln Cys Ser Arg Leu Arg Arg
50 55 60 65

CGG CGG CCC GAC GCG TCC GGC GGC GGT GGC AGC GGC GTC GCG TCC ATC 297
Arg Arg Pro Asp Ala Ser Gly Gly Gly Gly Ser Gly Val Ala Ser Ile
70 75 80

GTG TCG CGC TCG CTC TTC GAC CTG ATG CTG CTC CAC CGC AAC GAT GCG 345
Val Ser Arg Ser Leu Phe Asp Leu Met Leu Leu His Arg Asn Asp Ala
85 90 95

```
GCG TGC CCG GCC AGC AAC TTC TAC ACC TAC GAC GCC TTC GTC GCC GCC        393
Ala Cys Pro Ala Ser Asn Phe Tyr Thr Tyr Asp Ala Phe Val Ala Ala
        100                 105                 110

GCC AGC GCC TTC CCG GGC TTC GCC GCC GCG GGC GAC GCC GAC ACC AAC        441
Ala Ser Ala Phe Pro Gly Phe Ala Ala Ala Gly Asp Ala Asp Thr Asn
    115                 120                 125

AAG CGC GAG GTC GCC GCG TTC CTT GCG CAG ACG TCC CAC GAG ACC ACC        489
Lys Arg Glu Val Ala Ala Phe Leu Ala Gln Thr Ser His Glu Thr Thr
130                 135                 140                 145

GGC GGG TGG GCG ACG GCG CCC GAC GGC CCC TAC ACG TGG GGC TAC TGC        537
Gly Gly Trp Ala Thr Ala Pro Asp Gly Pro Tyr Thr Trp Gly Tyr Cys
                150                 155                 160

TTC AAG GAG GAG AAC GGC GGC GCC GGG CCG GAC TAC TGC CAG CAG AGC        585
Phe Lys Glu Glu Asn Gly Gly Ala Gly Pro Asp Tyr Cys Gln Gln Ser
            165                 170                 175

GCG CAG TGG CCG TGC GCC GCC GGC AAG AAG TAC TAC GGC CGG GGT CCC        633
Ala Gln Trp Pro Cys Ala Ala Gly Lys Lys Tyr Tyr Gly Arg Gly Pro
        180                 185                 190

ATC CAG CTC TCC TAC AAC TTC AAC TAC GGG CCG GCG GGG CAG GCC ATC        681
Ile Gln Leu Ser Tyr Asn Phe Asn Tyr Gly Pro Ala Gly Gln Ala Ile
    195                 200                 205

GGC GCC GAC CTG CTC GGC GAC CCG GAC CTC GTG GCG TCT GAC GCC ACC        729
Gly Ala Asp Leu Leu Gly Asp Pro Asp Leu Val Ala Ser Asp Ala Thr
210                 215                 220                 225

GTC TCC TTC GAC ACG GCC TTC TGG TTC TGG ATG ACG CCG CAG TCG CCC        777
Val Ser Phe Asp Thr Ala Phe Trp Phe Trp Met Thr Pro Gln Ser Pro
                230                 235                 240

AAG CCG TCG TGC AAC GCG GTC GCC ACC GGC CAG TGG ACG CCC TCC GCC        825
Lys Pro Ser Cys Asn Ala Val Ala Thr Gly Gln Trp Thr Pro Ser Ala
            245                 250                 255

GAC GAC CAG CGG GCG GGC CGC GTG CCG GGC TAC GGC GTC ATC ACC AAC        873
Asp Asp Gln Arg Ala Gly Arg Val Pro Gly Tyr Gly Val Ile Thr Asn
        260                 265                 270

ATC ATC AAC GGC GGG CTG GAG TGC GGC CAT GGC GAG GAC GAT CGC ATC        921
Ile Ile Asn Gly Gly Leu Glu Cys Gly His Gly Glu Asp Asp Arg Ile
    275                 280                 285

GCC GAC CGG ATC GGC TTC TAC AAG CGC TAC TGC GAC ATC CTC GGC GTC        969
Ala Asp Arg Ile Gly Phe Tyr Lys Arg Tyr Cys Asp Ile Leu Gly Val
290                 295                 300                 305

AGC TAC GGC GCC AAC TTG GAT TGC TAC AGC CAG AGG CCT TCG GCT CCT       1017
Ser Tyr Gly Ala Asn Leu Asp Cys Tyr Ser Gln Arg Pro Ser Ala Pro
                310                 315                 320
```

CCT AAG CTT CGC CTA CCT AGC TTC CAC ACA GTG ATA AAT AAT CAC 1062
Pro Lys Leu Arg Leu Pro Ser Phe His Thr Val Ile Asn Asn His
325 330 335

TGATGGAGTA TAGTTTACAC CATATCGATG AATAAAACTT GATCCGAATT CTCGCCCTAT 1122

AGTGAGTCGT ATTAGTCGAC AGCTCTAGA 1151

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 336 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Ala Leu Ala Val Val Ala Met Val Ala Arg Pro Phe Leu Ala
1 5 10 15

Ala Ala Val His Ala Glu Gln Cys Gly Ser Gln Ala Gly Gly Ala Val
20 25 30

Cys Pro Asn Cys Leu Cys Cys Ser Gln Phe Gly Trp Cys Gly Ser Thr
35 40 45

Ser Asp Tyr Cys Gly Ala Gly Cys Gln Ser Gln Cys Ser Arg Leu Arg
50 55 60

Arg Arg Arg Pro Asp Ala Ser Gly Gly Gly Gly Ser Gly Val Ala Ser
65 70 75 80

Ile Val Ser Arg Ser Leu Phe Asp Leu Met Leu Leu His Arg Asn Asp
85 90 95

Ala Ala Cys Pro Ala Ser Asn Phe Tyr Thr Tyr Asp Ala Phe Val Ala
100 105 110

Ala Ala Ser Ala Phe Pro Gly Phe Ala Ala Ala Gly Asp Ala Asp Thr
115 120 125

Asn Lys Arg Glu Val Ala Ala Phe Leu Ala Gln Thr Ser His Glu Thr
130 135 140

Thr Gly Gly Trp Ala Thr Ala Pro Asp Gly Pro Tyr Thr Trp Gly Tyr
145 150 155 160

Cys Phe Lys Glu Glu Asn Gly Gly Ala Gly Pro Asp Tyr Cys Gln Gln
165 170 175

Ser Ala Gln Trp Pro Cys Ala Ala Gly Lys Lys Tyr Tyr Gly Arg Gly
180 185 190

```
Pro Ile Gln Leu Ser Tyr Asn Phe Asn Tyr Gly Pro Ala Gly Gln Ala
        195                 200                 205

Ile Gly Ala Asp Leu Leu Gly Asp Pro Asp Leu Val Ala Ser Asp Ala
    210                 215                 220

Thr Val Ser Phe Asp Thr Ala Phe Trp Phe Trp Met Thr Pro Gln Ser
225                 230                 235                 240

Pro Lys Pro Ser Cys Asn Ala Val Ala Thr Gly Gln Trp Thr Pro Ser
                245                 250                 255

Ala Asp Asp Gln Arg Ala Gly Arg Val Pro Gly Tyr Gly Val Ile Thr
            260                 265                 270

Asn Ile Ile Asn Gly Gly Leu Glu Cys Gly His Gly Glu Asp Asn Arg
        275                 280                 285

Ile Ala Asp Arg Ile Gly Phe Tyr Lys Arg Tyr Cys Asp Ile Leu Gly
    290                 295                 300

Val Ser Tyr Gly Ala Asn Leu Asp Cys Tyr Ser Gln Arg Pro Ser Ala
305                 310                 315                 320

Pro Pro Lys Leu Arg Leu Pro Ser Phe His Thr Val Ile Asn Asn His
                325                 330                 335
```

That which is claimed is:

1. A DNA fragment comprising a monocotyledon promoter characterized as being responsive to physical and/or biological stress; wherein said DNA fragment is further characterized by the following relative pattern of expression in mature plants:

a low level of expression in leaves;

a moderate level of expression in plant stems; and

the highest level of expression in the plant roots and in the male and female parts of plant flowers.

2. A DNA fragment according to Claim 1 having substantially the same sequence as nucleotides 1836 to about 1884, as set forth in Sequence ID No. 1.

3. A DNA fragment according to Claim 2 further comprising, as part of the same contiguous fragment, substantially the same sequence as nucleotides 1 - 76, as set forth in Sequence ID No. 2.

4. A DNA fragment according to Claim 1 having substantially the same sequence as nucleotides 1810 to about 1884, as set forth in Sequence ID No. 1.

5. A DNA fragment according to Claim 4 further comprising, as part of the same contiguous fragment, substantially the same sequence as nucleotides 1 - 76, as set forth in Sequence ID No. 2.

6. A DNA fragment according to Claim 1 having substantially the same sequence as nucleotides 1724 to about 1884, as set forth in Sequence ID No. 1.

7. A DNA fragment according to Claim 6 further comprising, as part of the same contiguous fragment, substantially the same sequence as nucleotides 1 - 76, as set forth in Sequence ID No. 2.

8. A DNA construct comprising the monocotyledon promoter of Claim 1 operatively linked to at least one reporter gene.

9. A DNA construct according to Claim 8 wherein said reporter gene is selected from chloramphenicol acetyltransferase, β-glucuronidase, β-lactamase, or firefly luciferase.

10. A DNA construct comprising the monocotyledon promoter of Claim 1 operatively linked to at least one structural gene.

11. A DNA construct according to Claim 10 wherein said structural gene is selected from the Bacillus thuringensis toxin gene, genes encoding enzymes involved in phytoalexin biosynthesis, proteinase inhibitor genes, lytic enzyme genes, genes encoding fungal elicitors, or genes encoding inducers of plant disease resistance mechanisms.

12. Plant material containing the DNA construct of Claim 8.

13. Plant material containing the DNA construct of Claim 10.

14. A method for inducing the expression of heterologous, functional gene(s) in monocotyledon and dicotyledon plants, said method comprising:

subjecting the plant material of Claim 13 to conditions which induce transcription of said DNA construct.

15. A substantially pure protein having in the range of about 300 up to 350 amino acids, characterized by:

a hevein domain having in the range of about 20 - 40 amino acids, wherein said hevein domain is about 70 % homologous with respect to dicotyledonous chitinase hevein domains;

a glycine- and arginine-rich spacer region having in the range of about 6 up to 12 amino acids; and

a catalytic domain having in the range of about 240 - 280 amino acids, wherein said catalytic domain is about 77 % homologous with respect to dicotyledenous chitinase catalytic domains.

16. A protein according to Claim 15 having substantially the same amino acid sequence as set forth in Sequence ID No. 3.

17. A DNA encoding a protein according to Claim 15.

18. A DNA according to Claim 17 wherein said DNA further contains a readily detectable label.

19. A DNA according to Claim 18 wherein said label is selected from a radiolabeled molecule, a fluorescent molecule, a chemiluminescent molecule, an enzyme, a ligand, a toxin, or a selectable marker.

20. A method for the identification of novel chitinase genes, said method comprising

probing a nucleic acid library with at least a portion of the DNA of Claim 18 under hybridization conditions, and

selecting those clones of said library which hybridize with said probe.

FIG. 1A

```
                10                  30                  50
RICE      MRALAVVAMVARPF.....LAAAVHAEQCGSQAGGAVCPNCLCCSQFGWCGSTSDYCGAG
TOBACCO   ..............SL      LLSAS          R ASG    K     N N    P
POTATO1   .....TIFSLLFSLL      LN SGSN..VVHRPD L APG    K     N N    P
POTATO2     RHKE NF  YLLFSLLV VS AL QN    G  KA ASGQ   K     N N    S
BEAN      IWSVG  W L...L      VGGSYG     R     L  GGN              T    P

                70                  90                  110
RICE      .CQSQCSRLRRRRPDASGGGGSGVASIVSRSLFDLMLLHRNDAACPA.SNFYTYDAFVAA
TOBACCO   N       P.....GGPTPP   GDLG  I S M  Q  K     N  QG KG  S N  IN
POTATO1   N       P.....GGP PSGDLGGVI N M  Q  N     N  QGKN  S N  IS
POTATO2   N       P.....GGGPGP P GDLG AI N M  Q  K   ENS QG K  S N  IN
BEAN              .....GGPSPAPTDLSALI   T  Q  K     G     KG       I

                130 •               150                 170
RICE      ASAFPGFAAAGDADTNKREVAAFLAQTSHETTGGWATAPDGPYTWGYCFKEENGGAGPDY
TOBACCO   RS    GTS  TTAR   I    F                  A     WLR Q SP .
POTATO1   GS    GTT  ITAR   I                  PS      A     LR Q SP .
POTATO2   RS    GTS  INAR   I    F             S       A     LR R NP .
BEAN      K Y S GNT  TA R    I      G                   A     VR RNPST..
```

SUBSTITUTE SHEET

```
                190                 210                 230
RICE      CQQSAQWPCAAGKKYYGRGPIQLSYNFNYGPAGQAIGADLLGDPDLVASDATVSFDTAFW
TOBACCO    TP G     P R  F       I H Y    C R   V   NN      T PVI  KS L
POTATO1    TP S     P R  F       I H Y    C R   V   NN      T SVI  KS I
POTATO2    PP S     P R  F       I H Y    C R  AV   NN      T PVI  K  L
BEAN       SATP F   P QQ         I W Y   QC R   V   NK      T SVI  KS L

                250                 270                 290
RICE      FWMTPQSPKPSCNAVATGQWTPSADDQRAGRVPGYGVITNIINGGLECGHGEDDRIADRI
TOBACCO             HD II R O  SA RA N L  F               R T S VQ
POTATO1             HD I  R O  GA  A N    F                 S S VQ
POTATO2             HD II R N  SA RA N L  F               R T N VQ
BEAN          A     SHD I SR   SA VA R L    TV            R Q S VQ

                310                 330
RICE      GFYKRYCDILGVSYGANLDCYSQRPSAPP....KLRLPSFHTVINNH*
TOBACCO     R   S     P D    GN  SFGNGLLVDTM*
POTATO1     R   G     P D    GN  SFGNG    L ....VD  *
POTATO2     R   S    TP D    VN  WFGNALL  ..VDTL*
BEAN       F     L   G  N       T FGNS    L . SDLV SQ*
```

FIG. IB

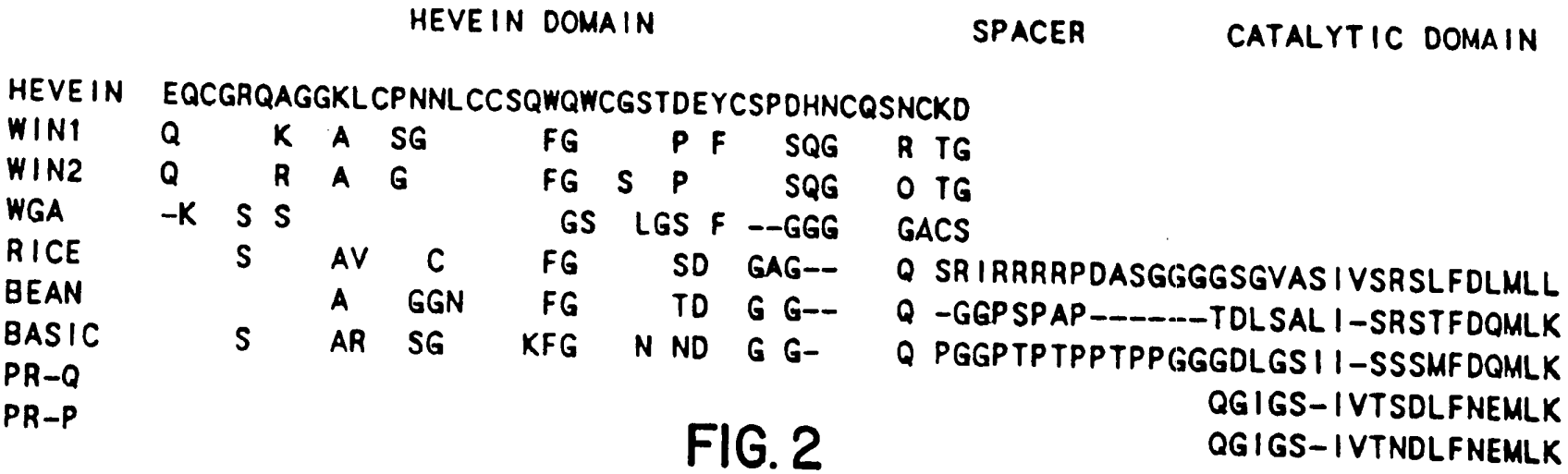

FIG. 2

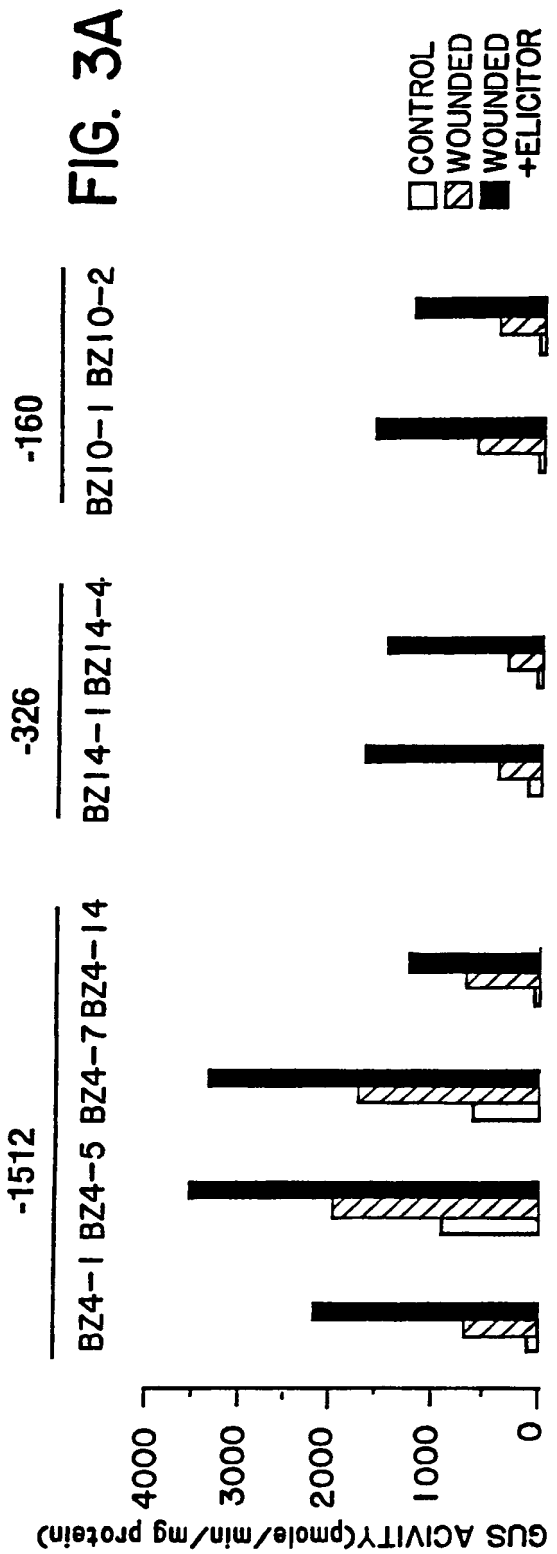
GUS ACTIVITY(pmole/min/mg protein)
4000
3000
2000
1000
0
-1512
BZ4-1 BZ4-5 BZ4-7BZ4-14
-326
BZ14-1BZ14-4
-160
BZ10-1 BZ10-2
CONTROL
WOUNDED
WOUNDED +ELICITOR

FIG. 3A

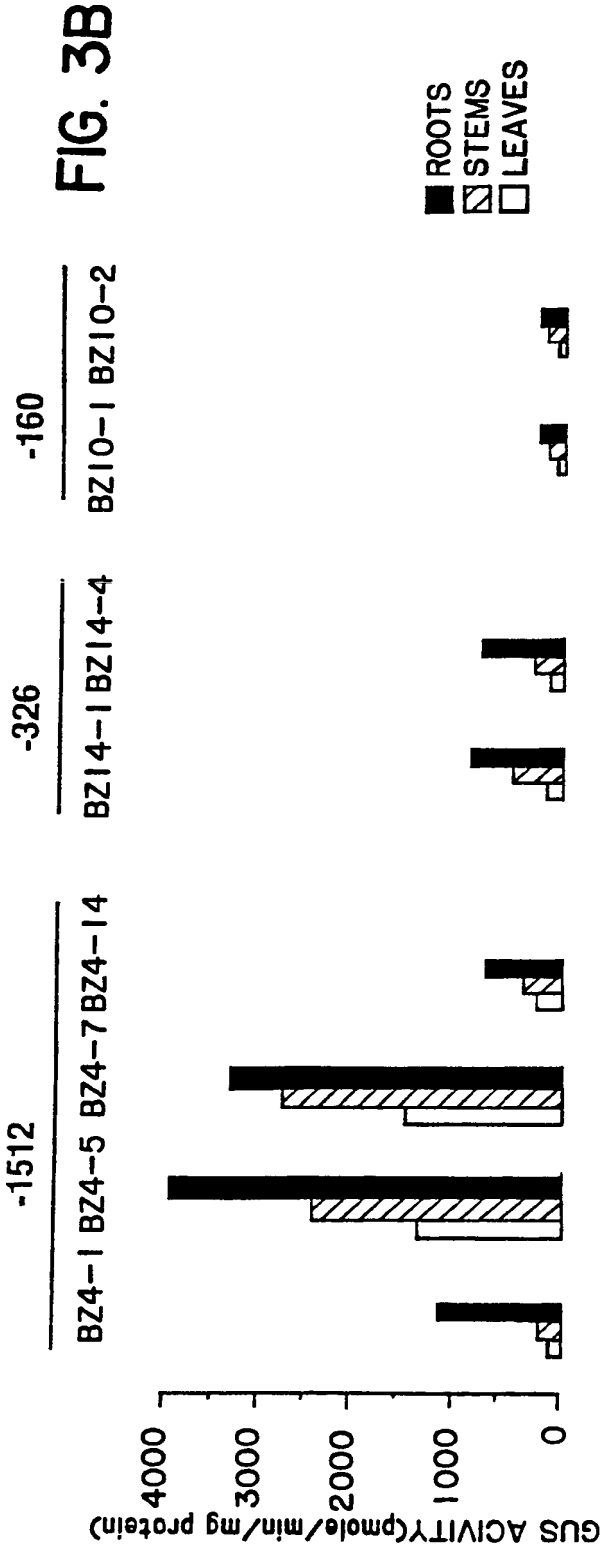
GUS ACTIVITY(pmole/min/mg protein)
4000
3000
2000
1000
0
-1512
BZ4-1 BZ4-5 BZ4-7BZ4-14
-326
BZ14-1BZ14-4
-160
BZ10-1 BZ10-2
ROOTS
STEMS
LEAVES

FIG. 3B

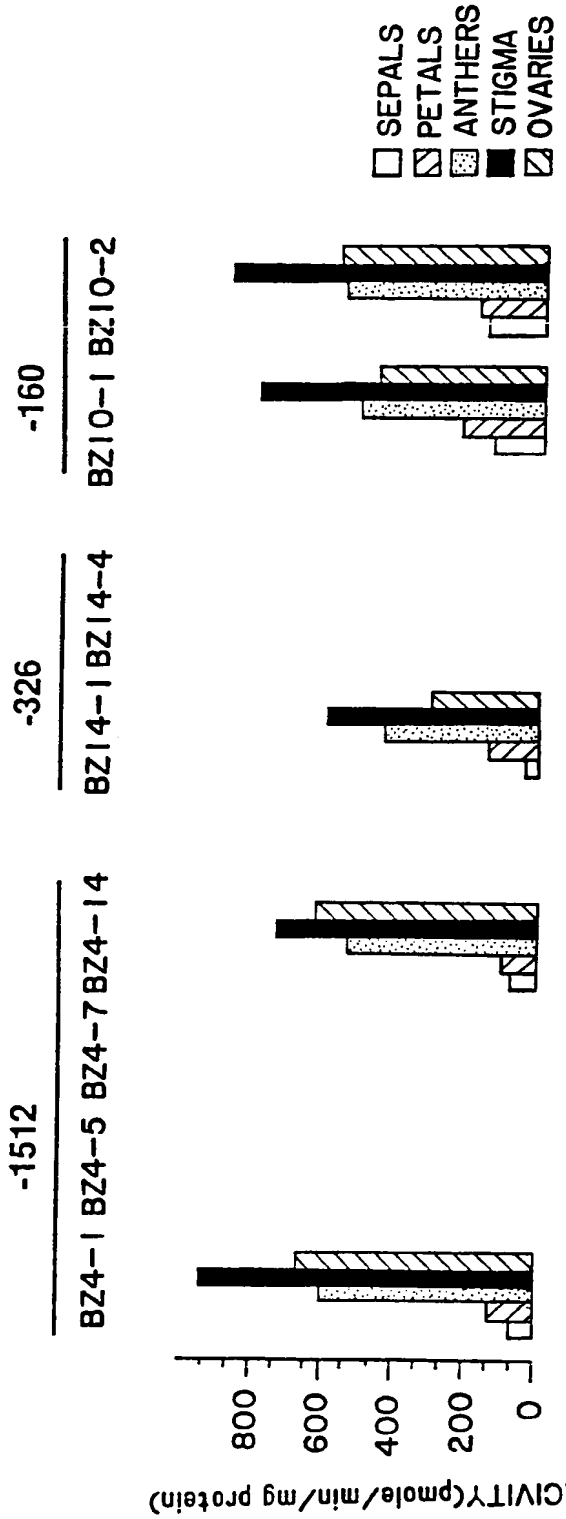

FIG. 3C

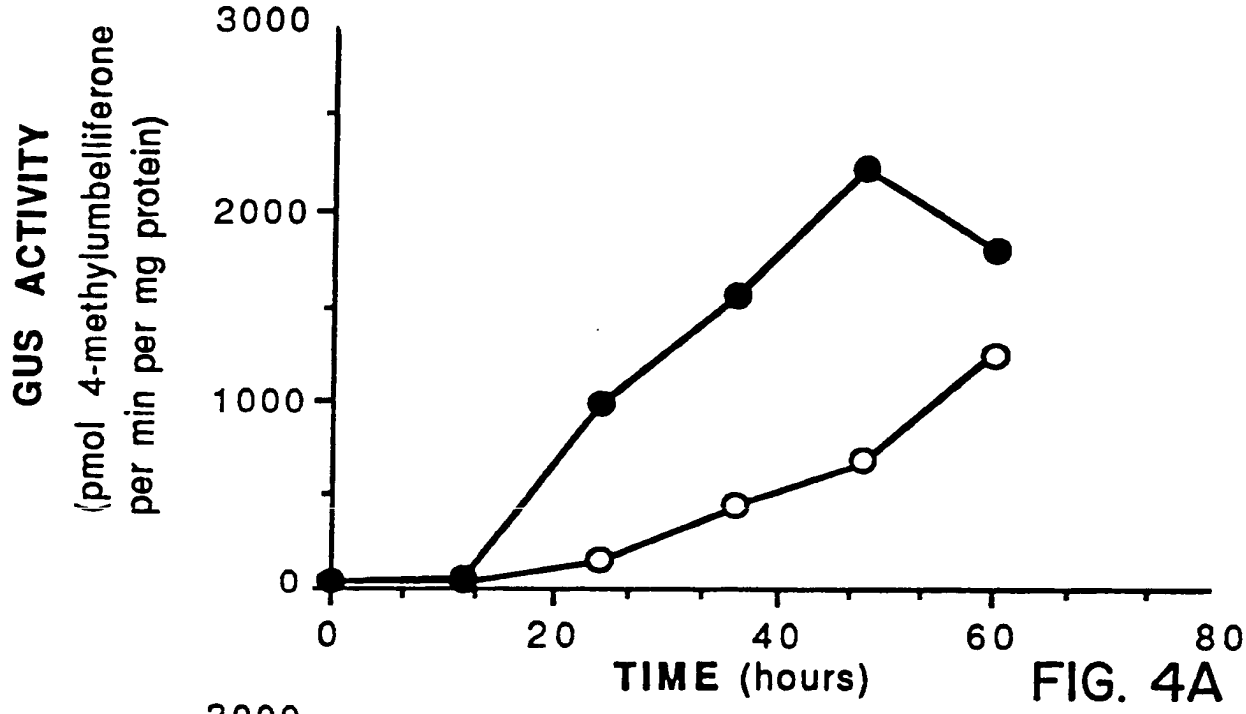
GUS ACTIVITY
(pmol 4-methylumbelliferone per min per mg protein)
3000
2000
1000
0
0
20
40
60
80
TIME (hours)

FIG. 4A

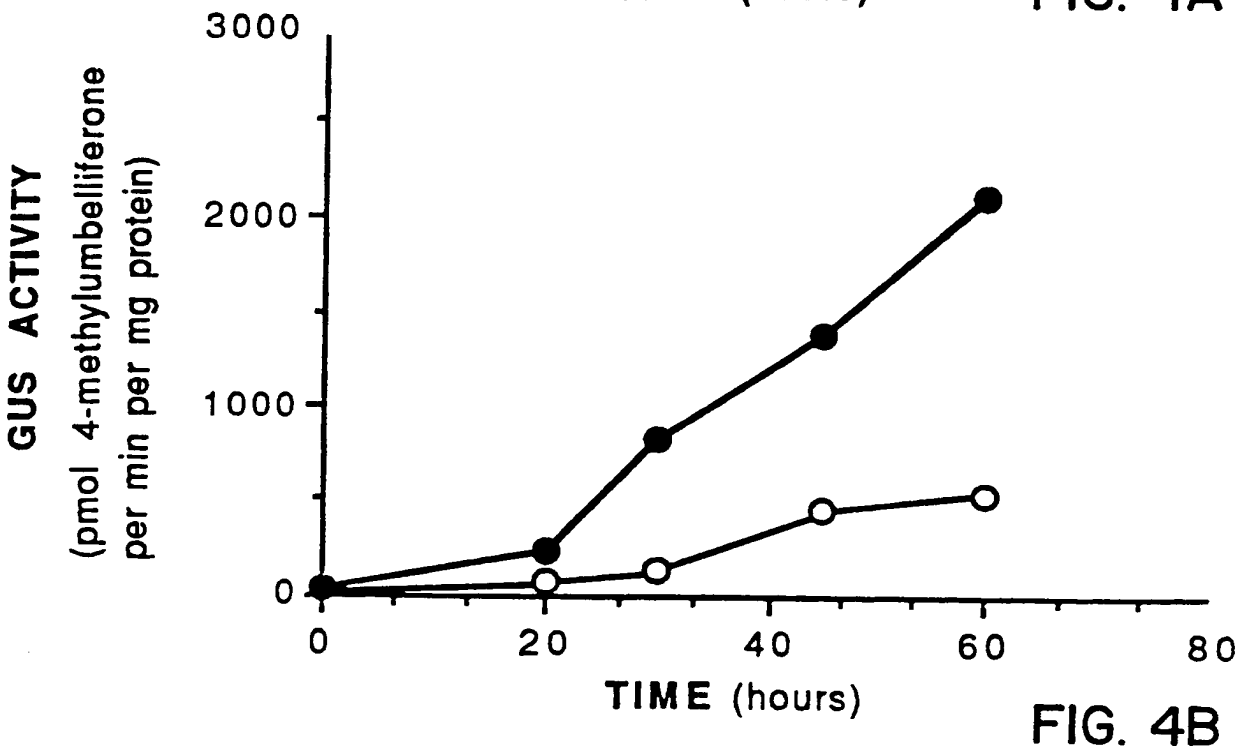
GUS ACTIVITY
(pmol 4-methylumbelliferone per min per mg protein)
3000
2000
1000
0
0
20
40
60
80
TIME (hours)

FIG. 4B

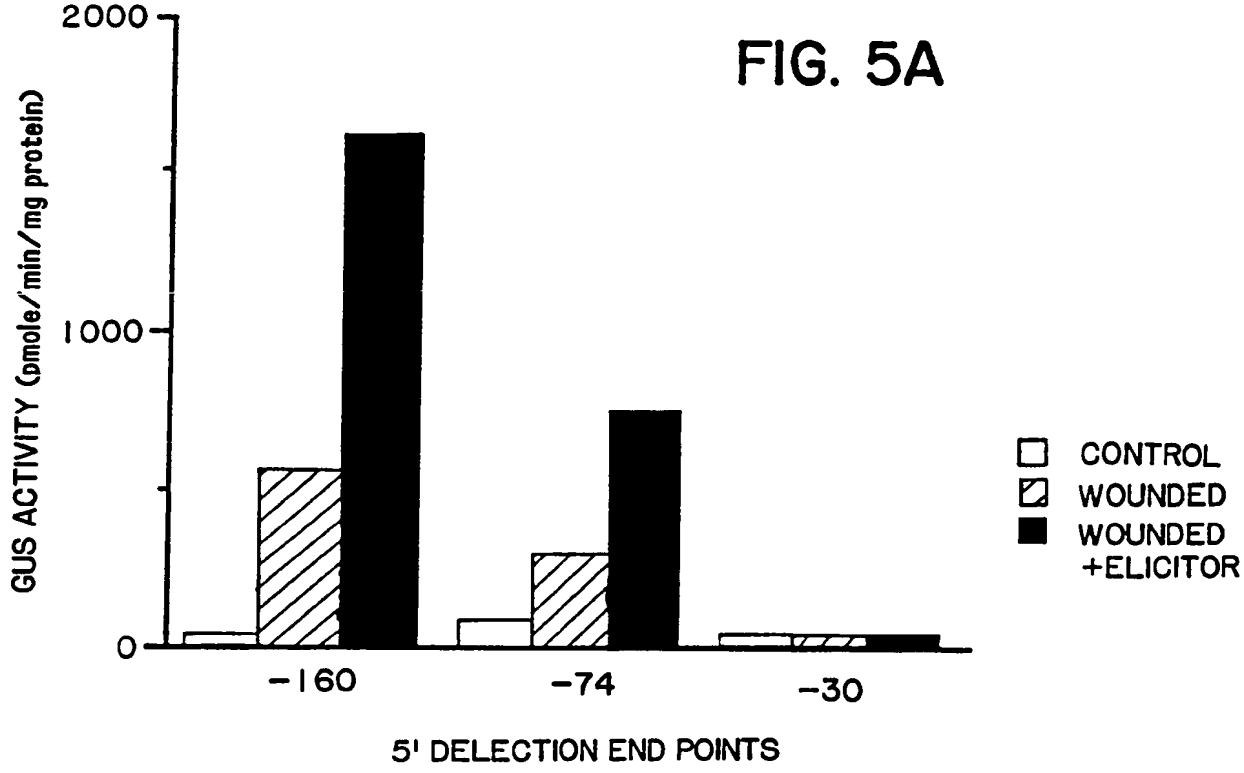

FIG. 5A

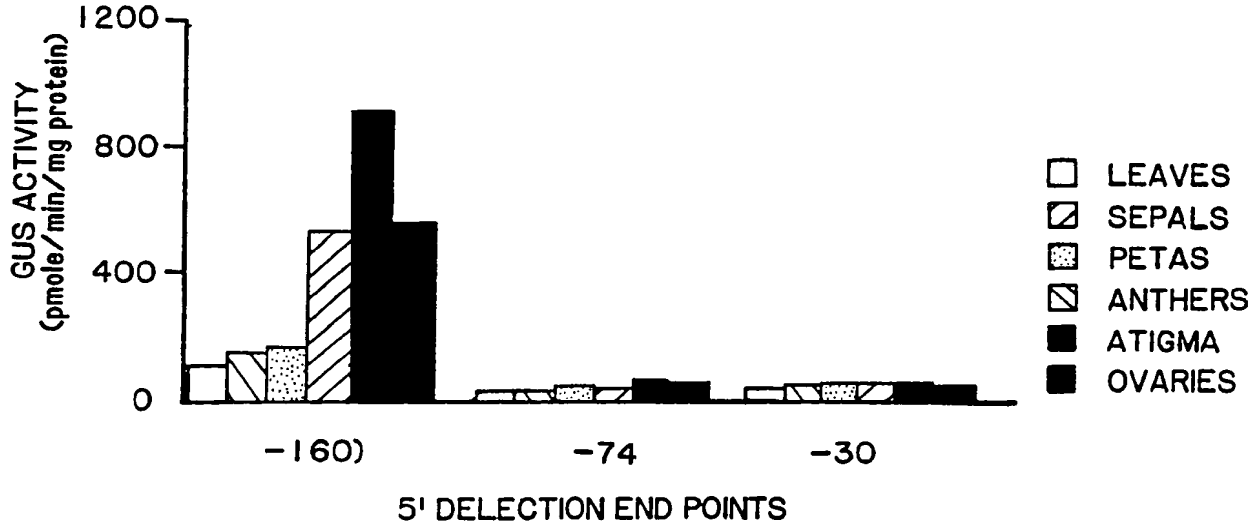

FIG. 5B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/04282

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/82, 15/56, 5/14, 9/24; C12Q 1/68; A01H 5/00; C07K 13/00
US CL :536/27; 435/320.1, 44, 69.1, 200, 6; 800/205; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/27; 435/320.1, 44, 69.1, 200, 6; 800/205; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	MGG, vol. 226, issued 21 June 1991, Zhu et al, "Isolation and characterization of a rice gene encoding a basic chitinase", pages 289-296, entire document.	1-20
X,P	Plant Science, vol. 76, issued 22 July 1991, Nishizawa et al, "Rice chitinase gene: cDNA cloning and stress-induced expression", p. 211-218, especially figure 2 and page 216.	17-20
X	Plant Molecular Biology, vol. 16, issued March 1991, Huang et al, "Nucleotide sequence of a rice genomic clone that encodes a class I endochitinase", p. 479-480, entire document.	1-7, 20
Y		8-19
Y	The Plant Cell, vol. 2, issued October 1990, Roby et al, "Activation of a bean chitinase promoter in transgenic tobacco plants by phytopathic fungi", p. 999-1007, especially abstract, page 100 first full paragraph, and figure 5.	8-14
Y	Physiologia Plantarum, vol. 79, issued July 1990, Jacobsen et al, "Characterization of two antifungal endochitinases from barley grain", p. 554-562, entire document.	15-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 AUGUST 1992	Date of mailing of the international search report 18 AUG 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer MARY E. MOSHER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

International application No.
PCT/US92/04282

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

EMBL-NEW 5, Genbank 71, Genbank-NEW 5, UEMBL 30-71, N-GeneSeq 6, APS, Biosis. Search terms: promoter, expression, monocot, maize, barley, wheat, rice, lily, onion, au= Zhu Q, au= Lamb C, hevein, chitinase, plant, plants, gene, genes, sequenc?, clon?; sequences corresponding to nucleotides 1836-1884, 1810-1884, 1724-1884 of seq. ID no. 1; sequence corresponding to sequence ID no. 3.